Novel roles for nasal epithelium in the pathogenesis of chronic rhinosinusitis with nasal polyps*

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Background: Airway epithelial cells have a well-accepted role in the regulation of local inflammatory processes in allergic and innate defence responses. However, their role the pathophysiology of chronic rhinosinusitis with nasal polyps (CRSwNP) is unclear. The objective was to investigate whether potential differences in the mRNA expression profile of nasal epithelia from healthy individuals and from CRSwNP patients would shed new light on disease mechanisms.

Methods: Primary epithelial cells from nasal polyps of 24 affected individuals and from middle turbinates of 9 healthy controls were obtained using magnetic beat assisted isolation and were used for expression profiling using the Human Genome U133 Plus 2.0 Genechip Array.

Results: Multiple gene probes corresponding to 27 genes showed an aberrant expression profile in polyp epithelial cells compared to healthy controls. Most of these genes are linked to pathogenic mechanisms seen in neoplasm formation, including changes in cell-cell adhesion, metabolic processes, cell cycle control, and differentiation. Remarkably, our data additionally suggest a role for maternally expressed genes in the pathogenesis of CRSwNP and reveal two distinct states of polyp epithelium that could not be linked to the presence or absence of atopy in patients or to the level of eosinophilia or neutrophilia of the polyp.

Conclusions: Our data suggest new roles for nasal epithelium in the pathogenesis of CRSwNP.

Key words: epithelium, chronic rhinosinusistis, nasal polyposis, microarray, expression profiling, epigenetics, neoplasm formation, polyp, pathogenesis

Introduction

Chronic rhinosinusitis with nasal polyps (CRSwNP) is a multifactorial chronic inflammatory disease that is characterized by a mass in the nasal cavity that most often originates from the paranasal sinuses[(1)]. It is a relative common disease with a prevalence of 4-5% in the general population with typical symptoms comprising nasal congestion, rhinorrhea, hyposmia, and facial pressure. Although the aetiology of CRSwNP is largely unknown, in people with asthma prevalence goes up to 6-15% and nasal polyps also share typical histological features. Polyps seem to resemble the bronchial mucosa of asthmatic patients with epithelial damage, goblet cell hyperplasia, thickening of the basement membrane, accumulations of extracellular matrix, fibrosis, and in the western population often eosinophil-dominated inflammation [(2)]. Given the active contribution of epithelium to the regulation of local inflammatory responses [(3)] in this manuscript we have explored the potential contribution of polyp epithelium to the pathogenesis of CRSwNP using micro-array expression profiling.

The potential contribution of nasal epithelium to the pathophysiology of CRSwNP has not been extensively explored, although expression of toll-like receptors and other innate immune response factors is well documented [(4)]. In allergic airway disease there is an established awareness of the role of epithelial cells

as an active participant in the regulation of local immune responses [5,6]. Epithelial cells are able to detect and respond to environmental signals through a wide variety of receptors, while epithelial integrity is still considered an important aspect in maintaining local homeostasis [7]. In addition to epithelial involvement in CRSwNP, it has been suggested that the interaction between nasal epithelium and myofibroblasts in CRSwNP could resemble the interaction between bronchial epithelium and smooth muscle cells [8]. In asthma the mutual and reciprocal activation within this epithelial mesenchymal trophic unit is thought to contribute to the pathophysiology of the disease. Indeed this link may help to explain the increased prevalence of CRswNP in asthma patients [1]. Other processes through which epithelium may directly or indirectly affect CRSwNP would be in the interaction with bacterial biofilms and orchestrations of (innate) immune responses [6].

Previously, we have investigated the role of nasal epithelium in allergic rhinitis to investigate intrinsic differences in expression profile that could contribute to the pathology of disease [9]. Using a similar approach in this study we identified affected genes in CRSwNP related to epithelial integrity, neoplasm formation, and glucose metabolism. Moreover we detected a novel epithelial dichotomy in CRSwNP that is not related to atopic status or to eosinophilic versus neutrophilic inflammation level of the polyp.

Material and Methods

Study design

This study was reviewed and approved by the medical ethical committee of the Academic Medical Center (06/062) and all participants signed informed consent. We included 24 CRSwNP patients and 9 controls (Table 1) that were generally healthy and did not have any auto-immune disorders or other relevant co-morbidities (e.g. aspirine intolerance) that could affect outcome measures. EP3OS criteria were used for inclusion of CRSwNP patients, while ARIA and GINA guidelines were used to establish allergic and asthma status. Atopy status was determined using the recommended GA2LEN panel of the 20 most common aeroallergens. The CRSwNP patients were operated because of severe symptoms of their disease while controls had non-secreting pituitary adenomas requiring endoscopic surgery and none of the participants had used local and/or systemic steroids four weeks prior to surgery.

Primary epithelial cell culture

Primary cells were obtained by digesting nasal biopsies or nasal polyps from the participants with 0.5 mg/mL collagenase 4 (Worthington Biochemical Corp., Lakewood, NJ, USA) for 1 hour in Hanks' balanced salt solution (Sigma-Aldrich, Zwijndrecht, the Netherlands). Epithelial cells were isolated using an anti-EpCAM microbead assisted cell isolation procedure (Miltenyi Biotec, Bergisch Gladbach, Germany) and grown in T25 cultured with BEGM in fully humidified air containing 5% CO_2 at 37°C to 80% confluence within 2 weeks.

Table 1. Demographics.

	Total number	Allergy	Asthma	Aspirin intolerance	Mean age (years)	Gender (M/F)
Healthy	9	0	0	0	54	7/2
CRSwNP	24	10	12	0	45	15/9

RNA extraction

Total RNA from each sample was extracted using Trizol (Life Technologies, Inc., Gaitersburg, MD, USA), followed by purification by nucleospin RNA II (Machery-Nagel, Düren, Germany). The RNA concentration was measured on the nanodrop ND-1000 (NanoDrop Technologies inc., Wilmington, DE, USA) and RNA quality was checked on the Agilent 2100 bio-analyzer (Agilent Technologies, Palo Alto, CA, USA).

Microarray data analysis and statistics

Human Genome U133 Plus 2.0 Genechip Array (Affymetrix inc., Santa Clara, CA, USA) was used in the analysis of the different expression patterns in diseased and healthy nasal epithelium. Technical handling of microarray experiments was performed at the MicroArray Department (MAD) of the University of Amsterdam and array images were acquired using a GeneChip Scanner 3000 (Affymetrix) and analyzed with Affymetrix GeneChip® Operating Software (Affymetrix).

The images and raw data passed manufacturers recommended quality criteria. GeneSpring v13 (Agilent technologies, Santa Clara, CA, USA) expression console was used to extract expression values, perform statistic testing, and analyse data. Expression levels were calculated using robust multi-array average (RMA) algorithm. Differences in expression were determined using unpaired statistical t tests with correction for multiple testing with a false discovery rate of 0.05 using the Westfall-Young procedure.

Immunohistochemistry

Section of snap frozen biopsies were stained for eosinophils (Clone BMK13 at 0.05 µg/mL, Monosan, Uden, the Netherlands) and neutrophils (Elastase at 2.2 µg/mL, DACO, Glastrup, Denmark) using Brightvision (Immunologic, Duiven, the Netherlands) as per manufacturer's instructions. All sections were examined by two independent observers blinded to the experimental conditions. The numbers of positively stained cells were counted in the lamina propria (per mm^2) at a final magnification of 200x. Statistical significance was determined with the Mann-Whitney U-test.

Table 2. Expression differences between epithelium from CRSwNP patients and healthy controls showing probe identity number (ID), fold change (FC), gene name, and chromosome location. N/A refers to not assigned probes.

Probe ID	Gene	FC	Gene name	Location
230835_at	KRTDAP	-7.49	keratinocyte differentiation-associated protein	chr19q13.12
206642_at	DSG1	-5.56	desmoglein 1	chr18q12.1
205625_s_at	CALB1	-4.08	calbindin 1, 28kDa	chr8q21.3
206004_at	TGM3	-4.05	transglutaminase 3	chr20q11.2
220225_at	IRX4	-3.21	iroquois homeobox 4	chr5p15.3
214536_at	SLURP1	-2.79	secreted LY6/PLAUR domain containing 1	chr8q24.3
205626_s_at	CALB1	-2.64	calbindin 1, 28kDa	chr8q21.3
212813_at	JAM3	-2.63	junctional adhesion molecule 3	chr11q25
221328_at	CLDN17	-2.56	claudin 17	chr21q22.11
231733_at	CARD18	-2.43	caspase recruitment domain family, member 18	chr11q22.3
212915_at	PDZRN3	-1.87	PDZ domain containing ring finger 3	chr3p13
217564_s_at	CPS1	-1.86	carbamoyl-phosphate synthase 1, mitochondrial	chr2q35
205637_s_at	SH3GL3	-1.84	SH3-domain GRB2-like 3	chr15q24
212730_at	SYNM	-1.83	synemin, intermediate filament protein	chr15q26.3
231148_at	IGFL2	-1.81	IGF-like family member 2	chr19q13.32
238022_at	CRNDE	-1.79	colorectal neoplasia differentially expressed	chr16q12.2
207023_x_at	KRT10	-1.70	keratin 10	chr17q21
213287_s_at	KRT10	-1.70	keratin 10	chr17q21
231240_at	DIO2	-1.51	deiodinase, iodothyronine, type II	chr14q24.2
242157_at	CHD9	-1.49	chromodomain helicase DNA binding protein 9	chr16q12.2
1555773_at	BPIFC	-1.46	BPI fold containing family C	chr22q12.3
227491_at	ELOVL6	-1.45	ELOVL fatty acid elongase 6	chr4q25
203700_s_at	DIO2	-1.43	deiodinase, iodothyronine, type II	chr14q24.2-3
231720_s_at	JAM3	-1.42	junctional adhesion molecule 3	chr11q25
244722_at	N/A	1.27	Not Assigned	N/A
232417_x_at	ZDHHC11	1.28	zinc finger, DHHC-type containing 11	chr5p15.33
200704_at	LITAF	1.33	lipopolysaccharide-induced TNF factor	chr16p13.13
200706_s_at	LITAF	1.37	lipopolysaccharide-induced TNF factor	chr16p13.13
238348_x_at	N/A	1.42	Not Assigned	N/A
202755_s_at	GPC1	1.44	glypican 1	chr2q35-37
209522_s_at	CRAT	1.56	carnitine O-acetyltransferase	chr9q34.1
216894_x_at	CDKN1C	1.58	cyclin-dependent kinase inhibitor 1C (p57, Kip2)	chr11p15.5
213182_x_at	CDKN1C	1.79	cyclin-dependent kinase inhibitor 1C (p57, Kip2)	chr11p15.5
219534_x_at	CDKN1C	1.83	cyclin-dependent kinase inhibitor 1C (p57, Kip2)	chr11p15.5
226403_at	TMC4	1.84	transmembrane channel-like 4	chr19q13.42
206088_at	LRRC37A3	1.90	leucine rich repeat containing 37, member A3	chr17q24.1
213348_at	CDKN1C	1.96	cyclin-dependent kinase inhibitor 1C (p57, Kip2)	chr11p15.5
228742_at	N/A	1.98	Not Assigned	N/A

Results

Deregulated expression of epithelial cancer related genes dominate the intrinsic differences between epithelia of healthy controls and CRSwNP patients

In total we identified 35 probe sets with a statistical significant different expression pattern (Table 2) between the concha epithelium of healthy individuals and the epithelium covering nasal polyps of patient with CRSwNP. These probe sets correspond to 27 different genes as the genes for calbindin 1 (CALB1), cyclin-dependent kinase inhibitor 1C (CDKN1C), iodothyronine deio-

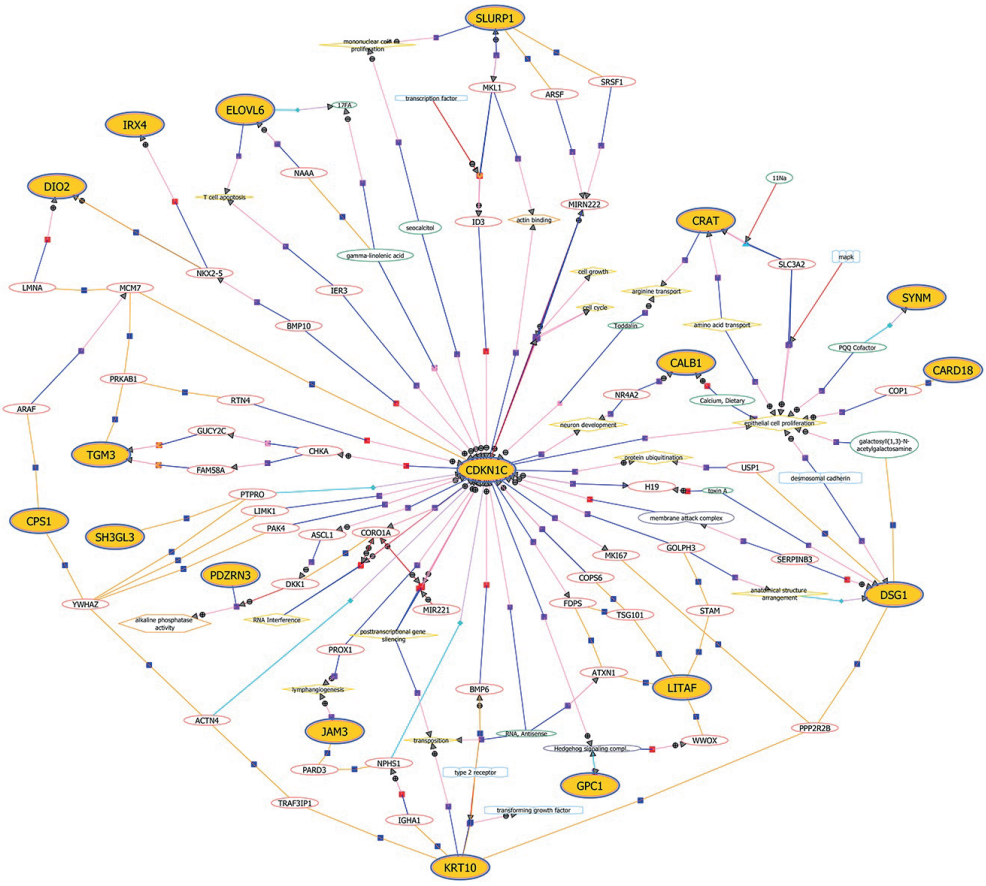

Figure 1. Shortest connect network of genes that are differentially expressed between healthy epithelium and polyp epithelium.

dinase 2 (DIO2), keratin 10 (KRT10), lipopolysaccharide-induced TNF factor (LITAF) and junctional adhesion molecule 3 (JAM3) are represented by multiple probes. The fold change for the probes belonging to each individual gene showed a conserved change of expression. The probes for CALB1 (-4.08 and -2.64 fold), JAM3 (-2.62 and -1.42 fold), KRT10 (-1.70 fold), and DIO2 (-1.43 and -1.51 fold) are all down-regulated in polyposis versus healthy controls, whereas in contrast the 4 probes representing CDKN1C (+1.58, +1.79, +1.83, and +1.96 fold) and the 2 probes representing LITAF (+1.33 and +1.37 fold) where all up-regulated. A selection of genes was used to validate the microarray expression data by correlating the expression level detected in the microarray with the expression level of an independent real time PCR of random samples. Indeed Table 3 shows a high level of correspondence in both data sets.

Eighteen of 27 deregulated genes form a shortest connection network (Figure 1). The most prominent of these genes is the maternally expressed tumour suppressor gene CDKN1C that previously has been linked to colon polyp formation [10]. Indeed, a substantial number of the affected genes have been linked to epithelial neoplasms and related biological processes. The deregulated expression of KRTDAP (keratinocyte differentiation-

Table 3 . Correlation coefficients showing high correspondence between microarray and RT-PCR expression levels

Gene	Correlation	p-value
CALB1	0.900	0.019
TGM3	0.913	0.015
DSG1	0.972	0.003
JAM3	0.946	0.008
SLURP1	0.988	0.001
KRTDAP	0.989	0.001

associated protein, -7.49 fold), DSG1 (desmoglein 1, -5.56 fold), TGM3 (transglutaminase 3, -4.05 fold), CLDN17 (claudin 17, -2.56 fold), JAM3 (-2.03 fold), SYNM (synemin, -1.83 fold), and KRT10 (keratin 10, -1.70 fold) shows changes in cell adhesion/ultra-structural complexes [11-17]. The deregulation of ELOVL6 (Elongation of Very Long Chain Fatty Acid Elongase 6, -1.45 fold), CRAT (Carnitine acetyltransferase, +1.56 fold), CRNDE (colorectal neoplasia differentially expressed, -1.79 fold), IGFL2 (IGF-like family member 2, -1.81 fold) point towards changes in insulin, glucose, and lipid metabolism [18-21]. Other proteins within our

Table 4A and B. Expression differences between two types epithelium from nasal polyps showing probe identity number (ID), fold change (FC), gene name, and chromosome location. N/A refers to not assigned probes.

Probe ID	FC	Gene	Gene name	Location
219975_x_at	-3.28	OLAH	oleoyl-ACP hydrolase	chr10p13
233126_s_at	-3.09	OLAH	oleoyl-ACP hydrolase	chr10p13
222945_x_at	-2.72	OLAH	oleoyl-ACP hydrolase	chr10p13
205597_at	-2.72	SLC44A4	solute carrier family 44, member 4	chr6p21.3
225496_s_at	-2.53	SYTL2	synaptotagmin-like 2	chr11q14
221011_s_at	-2.47	LBH	limb bud and heart development	chr2p23.1
1555203_s_at	-2.43	SLC44A4	solute carrier family 44, member 4	chr6p21.3
221523_s_at	-2.42	RRAGD	Ras-related GTP binding D	chr6q15-q16
232914_s_at	-2.32	SYTL2	synaptotagmin-like 2	chr11q14
203892_at	-2.30	WFDC2	WAP four-disulfide core domain 2	chr20q13.12
211163_s_at	-2.28	TNFRSF10C	TNF receptor superfamily, member 10c	chr8p22-p21
214453_s_at	-2.23	IFI44	interferon-induced protein 44	chr1p31.1
218885_s_at	-2.19	GALNT12	N-acetylgalactosaminyltransferase 12	chr9q22.33
206222_at	-2.16	TNFRSF10C	TNF receptor superfamily, member 10c	chr8p22-p21
221524_s_at	-2.10	RRAGD	Ras-related GTP binding D	chr6q15-q16
223551_at	-2.04	PKIB	protein kinase inhibitor beta	chr6q22.31
223423_at	-1.97	GPR160	G protein-coupled receptor 160	chr3q26.2-q27
235911_at	-1.95	MFI2	melanoma associated antigen p97	chr3q28-q29
205259_at	-1.88	NR3C2	nuclear receptor subfamily 3, member C2	chr4q31.1
210538_s_at	-1.74	BIRC3	baculoviral IAP repeat containing 3	chr11q22
212503_s_at	-1.72	DIP2C	DIP2 disco-interacting protein 2 homolog C	chr10p15.3
212686_at	-1.71	PPM1H	protein phosphatase, Mg/Mn dependent 1H	chr12q14.1
205278_at	-1.70	GAD1	glutamate decarboxylase 1	chr2q31
223784_at	-1.70	TMEM27	transmembrane protein 27	chrXp22
234689_at	-1.69	PTCHD4	patched domain containing 4	chr6p12.3
227909_at	-1.62	LINC086-87	long intergenic non-protein coding RNA 86-87	chrXq26.3
204671_s_at	-1.60	ANKRD6	ankyrin repeat domain 6	chr6q14.2-q16.1
204624_at	-1.59	ATP7B	ATPase, Cu++ transporting, beta polypeptide	chr13q14.3
202161_at	-1.56	PKN1	protein kinase N1	chr19p13.12
209114_at	-1.56	TSPAN1	tetraspanin 1	chr1p34.1
209499_x_at	-1.55	TNFSF12-13	TNF superfamily, member 12-13	chr17p13-p13.1
242931_at	-1.55	LONRF3	LON peptidase N-term domain and ring finger 3	chrXq24
214667_s_at	-1.54	TP53I11	tumor protein p53 inducible protein 11	chr11p11.2
202150_s_at	-1.53	NEDD9	neural expressed, developmental down-regul. 9	chr6p25-p24
207949_s_at	-1.49	ICA1	islet cell autoantigen 1, 69kDa	chr7p22
203332_s_at	-1.49	INPP5D	inositol polyphosphate-5-phosphatase	chr2q37.1
212325_at	-1.49	LIMCH1	LIM and calponin homology domains 1	chr4p13
236656_s_at	-1.47	N/A	uncharacterized LOC100288911	chr2p22.3
244486_at	-1.47	N/A	Not Assigned	N/A
209500_x_at	-1.42	TNFSF12-13	TNF superfamily, member 12-13	chr17p13-p13.1
225548_at	-1.38	SHROOM3	shroom family member 3	chr4q21.1
204276_at	-1.38	TK2	thymidine kinase 2, mitochondrial	chr16q22-23
205298_s_at	-1.38	BTN2A2	butyrophilin, subfamily 2, member A2	chr6p22.1
219952_s_at	-1.37	MCOLN1	mucolipin 1	chr19p13.2

Probe ID	FC	Gene	Gene name	Location
229253_at	-1.36	THEM4	thioesterase superfamily member 4	chr1q21
213045_at	-1.34	MAST3	microtubule associated ser/thr kinase 3	chr19p13.11
212745_s_at	-1.31	BBS4	Bardet-Biedl syndrome 4	chr15q22.3-q23
241353_s_at	-1.29	N/A	Not Assigned	N/A
224998_at	-1.28	CMTM4	CKLF-like MARVEL domain containing 4	chr16q21-q22.1
209166_s_at	-1.28	MAN2B1	mannosidase, alpha, class 2B, member 1	chr19cen-q13.1
Probe ID	**FC**	**Gene**	**Gene name**	**Location**
230835_at	3.78	KRTDAP	keratinocyte differentiation-associated protein	chr19q13.12
1554179_s_at	3.45	LYNX1	Ly6/neurotoxin 1	chr8q24.3
203691_at	2.72	PI3	peptidase inhibitor 3, skin-derived	chr20q13.12
224329_s_at	2.70	CNFN	cornifelin	chr19q13.2
41469_at	2.57	PI3	peptidase inhibitor 3, skin-derived	chr20q13.12
235272_at	2.33	SBSN	suprabasin	chr19q13.13
1554253_a_at	2.27	CERS3	ceramide synthase 3	chr15q26.3
204803_s_at	2.18	RRAD	Ras-related associated with diabetes	chr16q22
206517_at	2.08	CDH16	cadherin 16, KSP-cadherin	chr16q22.1
204802_at	2.07	RRAD	Ras-related associated with diabetes	chr16q22
1553077_at	1.94	SDR9C7	short chain dehydrogenase/reductase 9C 7	chr12q13.3
213796_at	1.91	SPRR1A	small proline-rich protein 1A	chr1q21-22
214536_at	1.79	SLURP1	secreted LY6/PLAUR domain containing 1	chr8q24.3
214549_x_at	1.75	SPRR1A	small proline-rich protein 1A	chr1q21-22
1570005_at	1.58	N/A	Not Assigned	N/A
227854_at	1.50	N/A	Not Assigned	N/A
229215_at	1.49	ASCL2	achaete-scute complex homolog 2	chr11p15.5
226177_at	1.48	GLTP	glycolipid transfer protein	chr12q24.11
243388_at	1.48	N/A	Not Assigned	N/A
202917_s_at	1.48	S100A8	S100 calcium binding protein A8	chr1q21
239230_at	1.48	HES5	hairy and enhancer of split 5 (Drosophila)	chr1p36.32
1555019_at	1.46	CDHR1	cadherin-related family member 1	chr10q23.1
211076_x_at	1.37	ATN1	atrophin 1	chr12p13.31
1564658_at	1.33	NAT16	N-acetyltransferase 16 (GCN5-related, putative)	chr7q22.1
206671_at	1.26	SAG	S-antigen; retina and pineal gland (arrestin)	chr2q37.1
234761_at	1.25	N/A	Not Assigned	N/A
1556753_s_at	1.23	N/A	Not Assigned	N/A
1554044_a_at	1.21	MRAP	melanocortin 2 receptor accessory protein	chr21q22.1

deregulated gene set that also have been previously reported to be affected in epithelial cancers include CHD9 (chromodomain helicase DNA binding protein 9, -1.49 fold), IRX4 (iroquois homeobox 4, -3.21 fold), SLURP1 (secreted LY6/PLAUR domain containing 1, -2.79 fold), CARD18 (caspase recruitment domain family member 18, -2.43 fold), CPS1 (mitochondrial carbamoyl-phosphate synthase 1, -1.86), DIO2 (-1.47 fold), and GPC1 (glypican 1, +1.44 fold) [22-28].

Two distinct epithelial expression profiles in nasal polyps

A more detailed representation using hierarchical clustering shows individual expression levels of 35 probes in healthy turbinates and nasal polyps. In addition to the overall differences of up- and down-regulated genes, this analysis suggests that epithelium of nasal polyps can be divided into two clusters as suggested by the tree cluster structure for the different polyps (Figure 2). The differences between the two expression clusters in CRSwNP seem largely dominated, but not uniquely, by expression of KRTDAP that has previously been reported to be involved in squamous cell differentiation and stratification of epithelia [11].

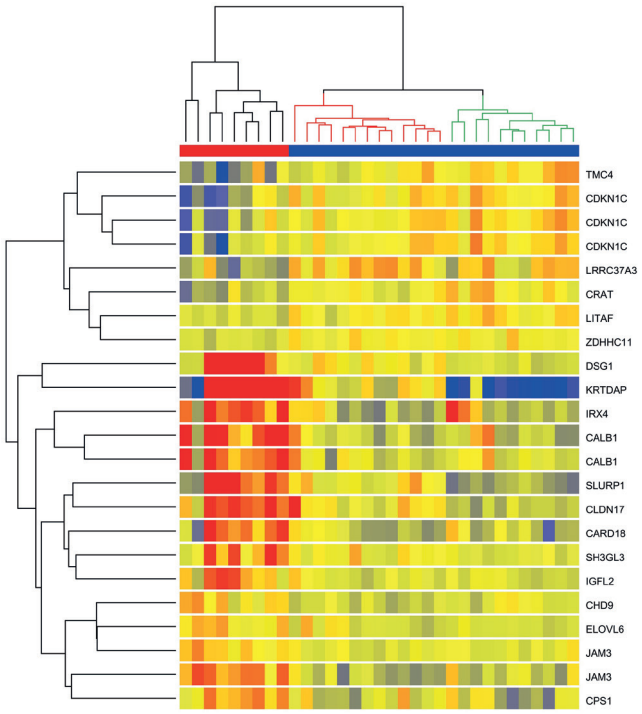

Figure 2. Hierarchical clustering (for probes on the left hand side and for individuals on the top) of the differentially expressed genes between healthy epithelium (black bar) and polyps epithelium (grey bar) showing relative expression (high in red and low in blue). Within the CRSwNP group the two sub-clusters are indicated by the red en green colouring of the dendrogram.

To explore the differences between these potential groups of polyps we compared the expression profiles between these two groups. Indeed, in addition to the differences suggested by expression profiles of KRTDAP, the direct comparison of expression profiles of both groups of polyps reveals 77 probes that correspond to 68 genes that are differentially expressed (Table 4A/4B). The expression profile strengthens the notion of two types of differentiated epithelia, as in addition to KRTDAP we now also detect differential expression of the structural protein CNFN (cornifelin), SPRR1A (small proline-rich protein 1A), and SBSN (suprabasin) that together with HES5 (hairy and enhancer of split 5), RRAD (Ras-related associated with diabetes), SDR9C7 (short chain dehydrogenase/reductase 9C7, ASCL2 (achaete-scute complex homolog 2), GLTP (glycolipid transfer protein), S100A8 (S100 calcium binding protein A8), SAG (S-antigen), NR3C2 (nuclear receptor subfamily 3, member C2), BIRC3 (baculoviral IAP repeat containing 3), GAD1 (glutamate decarboxylase 1), ATP7B (ATPase 7B), TSPAN1 (tetraspanin 1), and NEDD9 (neural expressed, developmental down-regulated 9) are typically deregulated in squamous cell epithelial carcinoma's [29-44].

Gene set enrichment analysis furthermore identifies deregulated expression of TNFSF12-13 (TNF superfamily, member 12-13), TNFRSF10C (TNF receptor superfamily, member 10C), and BIRC3 to TWEAK- (wikipathway 2036, p=0.0018) and TRAIL-mediated apoptosis (wikipathway 1772, p=0.0065), as important differences between the two types of epithelia. When we further examine the genes that are most affected we note deregulation of LYNX1 (Ly-6/neurotoxin-like protein 1 or SLURP2) and SLC44A4 (solute carrier family 44, member 4 or CTL4) that, like the previously identified SLURP1, are involved in acetylcholine-mediated neurogenic inflammation [45,46]. These two processes (apoptosis and neurogenic inflammation) are indeed often affected in different forms of carcinomas. Their relevance is further supported by representatives of these processes that feature in a shortest connect pathway of all affected genes (Figure 3) centred around S100A8 and IFI44 (interferon-induced protein 44). The differences in expression of these genes and processes, however, are not related to differences in level of eosinophils (2.5 cells/mm^2 in ciliated epithelium, with a range of 0 – 14.9 cells/mm^2, versus 6.4 cells/mm^2 in squamous epithelium, with a range of 0 – 39.2 cells/mm^2; p = 0.143), neutrophils (5.3 cells/mm^2 in ciliated epithelium, with a range of 0 – 28.7 cells/mm^2, versus 19.2 cells/mm^2 in squamous epithelium with a range of 0.7– 35.1 cells/mm^2; p = 0.126), nor in prevalence of atopic comorbidities (p = 0.616) between the two polyp groups (data not shown).

Discussion

In this manuscript we provide a detailed overview of intrinsic expression differences between CRSwNP epithelium and healthy control epithelium. The outcome shows that most of these differences are related to changes in ultra-structural organisation, differentiation state, and processes linked to (epithelial) neoplasm formation. In addition to these processes, the data also identified an acetylcholine-centred inflammatory process as potential pathological mechanism in CRSwNP and also suggest a role for epigenetical expression regulation. Moreover, at the epithelial level CRSwNP polyps seem to divide into two groups that are not linked to the presence or absence of allergy or the level of inflammation.

Pathological concepts in CRSwNP

The deregulated expression of DSG1, JAM3, TGM3, SYNM, and CLDN17 shows that the epithelial integrity defects seen in vivo are cell intrinsic. In allergic disease intrinsic barrier dysfunction is thought to directly contribute to the pathophysiology, as it would facilitate access of allergen. To what extend the changes in adhesion/tight junction proteins play a direct role in the pathology of CRSwNP remains to be explored. On one hand changes in the composition of these adhesion structures could affect their functionality or these changes are part of a compensatory mechanism that tries to counteract the negative effect local inflammatory processes may have on epithelial integrity. On the other hand the loose epithelial structure may in contrast

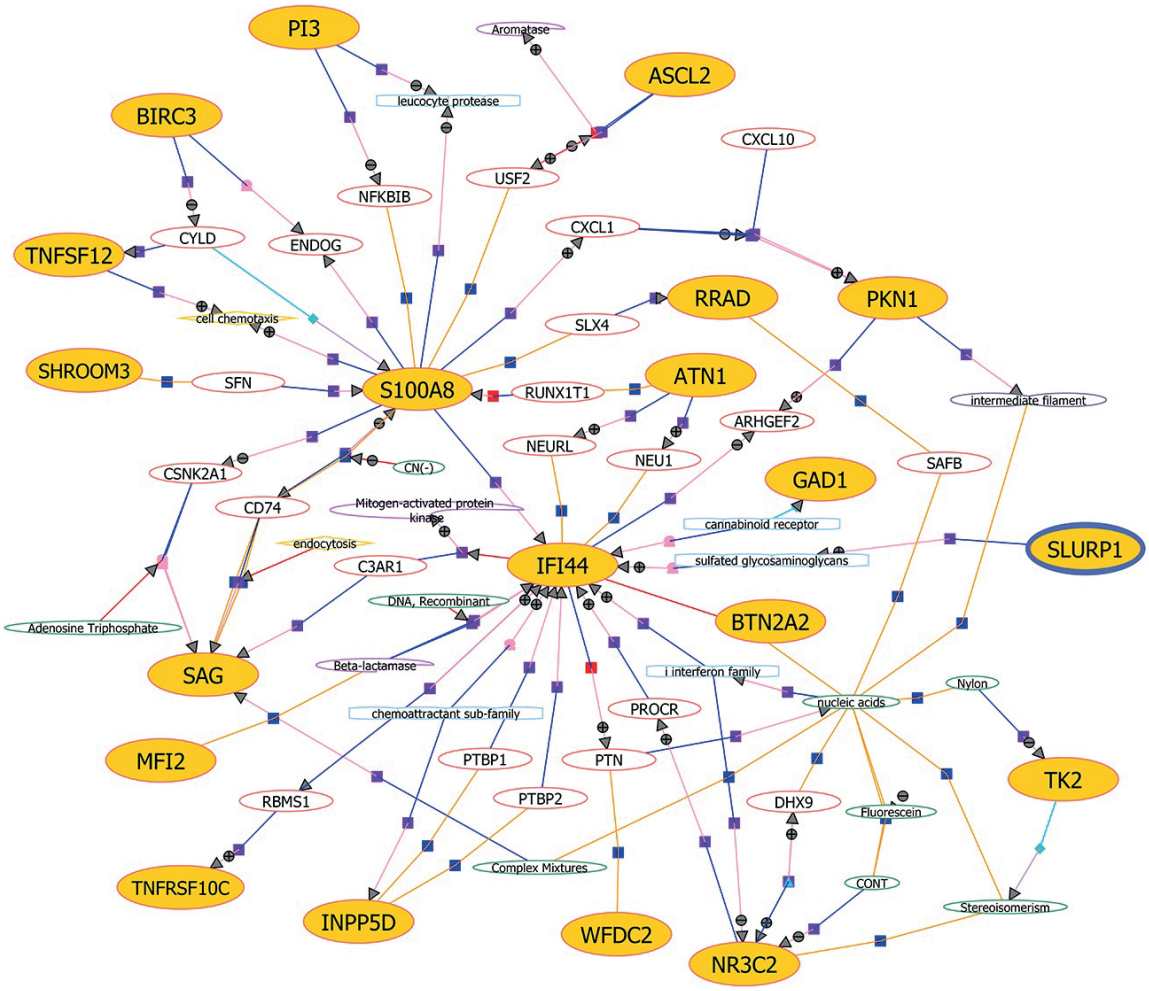

Figure 3. Shortest connect network of genes that are differentially expressed between the two types of polyp epithelium.

even help to drain local inflammation and mitigates its effect. Although the nasal polyps we have studied in this manuscript are not malignant per se, the changes in ELOVL6, CHD9, ASCL2, CRNDE, IGFL2, IRX4, SLURP1, CARD18, CPS1, DIO2, GPC1 and CDKN1C have been linked to cellular transformation [10,18-20,22-28]. Interestingly, ELOVL6, DIO2, IGFL2, CALB1, and CRNDE have been linked to metabolic changes seen in a variety of cancers. The most relevant might well be the maternally expressed tumour suppressor gene CDKN1C. Deregulated expression of this gene has been linked to multiple forms of cancer, and mutations in this gene underlie Beckwith-Wiedemann Syndrome [10]. A dedicated locus on chromosome 11 (chr11p15.5) is home to the archetypes of epigenetically regulated and growth affecting gene IGF2 (insulin growth factor 2) and the non-coding RNA H19.55 Indeed in addition to CDKN1C also the deregulated and imprinted gene ASCL2 (achaete-scute complex homolog 2) is located in this region [156].

Neurogenic inflammation

Although neurogenic inflammation has been studied in relationship to inflammation and neoplasm formation, it has not been extensively considered in CRSwNP. Recently it has become clear that epithelial cells produce and respond to acetylcholine [58]. In this light we can see the deregulated expression of SLURP1, Lynx1, and SLC44A4. SLURP1 acts as a potential negative regulator of acetylcholine receptor mediated signalling. In this way it limits epithelial transformation induced by nicotine contained within cigarette smoke, as nicotine is a ligand for the acetylcholine receptor [68] LYNX1 belongs to the same family of proteins and has a similar function as SLURP1. SLC44A4 is a acetylcholine transporter seems specific for acetylcholine secretion [46,59]. The precise role of epithelial-centred neurogenic inflammation is not yet fully established. One aspect seems to focus on signalling between adjacent epithelial cells and inflammatory cells in the nasal cavity [58].

Additional considerations

Even though the CRSwNP patients included in our study display a mix of varying allergic and/or asthmatic co-morbidities, none of the epithelial deregulated genes show a direct link to allergy. This suggests that at least from an epithelial perspective, allergy is an epiphenomenon and that the epithelial differences we have previously observed in allergic rhinitis do not play a common role in CRSwNP [9].

Cause or effect remains a difficult issue when trying to interpret the differences between the epithelia of CRSwNP patients and health controls. One caviate would be the choice of middle turbinate as control where polyps arise in the ethmoidal region. Unfortunately, obtaining the corresponding region from a healthy control is relatively hard so we opted to use this common control in CRSwNP research. Secondly, in active disease the nasal epithelium will be exposed to many inflammatory triggers that could affect the expression profile. While on one hand these effects could play an important role in the pathological conditions of CRSwNP, on the other hand these differences could just point to innocent bystander effects. We have tried to mitigate some of these aspects by limited culturing of epithelial cells rather than analysing the expression profile in situ or directly after isolation. Although, this approach also allows us to obtain a pure epithelial population, the culture procedure itself may have undocumented effects on the expression profile, and furthermore these effects may differ between diseased and healthy epithelium. For instance, the use of standard submerged culture conditions does not allow the formation of differentiated epithelium which will have consequences for the kind of cell-cell contacts that can be formed. Just like presence of culture media components like corticosteroids will have consequences. Despite these drawbacks, the advantage of this approach of being able to look at stablely transmitted differences induced by CRSwNP in airway epithelial cells and all cells are cultured under identical conditions. We are somewhat reassured that the deregulated genes we have identified can be linked to processes that previously have been hypothesized to have some role in the pathophysiology of the disease [1]. The re-affirmation of older hypotheses also gives credence to observations that seem to point to a potential involvement of neurogenic inflammation in the pathophysiology of CRSwNP.

The differences in epithelial expression profile come from our specific patient population. All of our CRSwNP patients have recurrent disease, despite optimal medical treatment, have had multiple previous surgeries, and would as be as far removed from primary polyposis as can be imagined. Although, it is these patients that need our help the most, it may well be that the initial changes that would lead to the formation of nasal polyps could be different from the differences we have discussed here.

Conclusions

In conclusion, our data identified differences in expression profile between epithelia of CRSwNP patients and that of healthy controls. Although these differences might not necessarily be the cause of the disease, they do point towards new processes that could play a role in the pathophysiology and open new avenues for future research.

Authorship contribution

MEC, KK, and ABR acquired the data, MEC, AHZ, and CMvD analysed the data, ABR, MEC, DvE, and EJdeG performed the experiments, KK, ABR, WF, and CMvD conceived the work, MEC, WF, and CMvD interpreted the results and drafted the manuscript, all authors revised the manuscript and all approved the final version.

Conflict of interest

None of the authors has anything to disclose related to this manuscript.

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